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Research Paper

Selective chemical inactivation of AAA proteins reveals distinct functions of proteasomal ATPases

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Abstract

Background: The 26S proteasome contains six highly related ATPases of the AAA family. We have developed a strategy that allows selective inhibition of individual proteasomal ATPases in the intact proteasome. Mutation of a threonine in the active site of Sug1/Rpt6 or Sug2/Rpt4 to a cysteine sensitizes these proteins to inactivation through alkylation by the sulfhydryl modifying agent NEM. Using this technique the individual contributions of Sug1 and Sug2 to proteasome function can be assessed.

Results: We show that both Sug1 and Sug2 can be selectively alkylated by NEM in the context of the intact 26S complex and as predicted by structural modeling, this inactivates the ATPase function. Using this technique we demonstrate that both Sug 1 and 2 are required for full peptidase activity of the proteasome and that their functions are not redundant. Kinetic analysis suggests that Sug2 may have an important role in maintaining the interaction between the 19S regulatory complex and the 20S proteasome. In contrast, inhibition of Sug1 apparently decreases peptidase activity of the 26S proteasome by another mechanism.

Conclusions: These results describe a useful technique for the selective inactivation of AAA proteins. In addition, they also demonstrate that the functions of two related proteasomal AAA proteins are not redundant, suggesting differential roles of proteasomal AAA proteins in protein degradation.

Keywords: Chemical inactivation; AAA proteins; Proteasomal ATPases

1. Introduction

The AAA (ATPases Associated with diverse cellular Activities) family is a large group of proteins that contain one or two copies of a conserved 230 amino acid motif called the AAA module [1]. They are involved in a wide variety of processes including vesicle fusion, proteolysis, transcriptional regulation, peroxisomal biogenesis, cell cycle regulation, meiosis, microtubule severing and chaperone-like functions [2,1]. Within the well-conserved AAA module are Walker A and B nucleotide binding motifs [3], and ATPase activity or ATP dependence has been demonstrated for many of these proteins (e.g. [4–6]). While the AAA module is highly conserved in all proteins of the family, the proteins share less homology outside of the AAA motif. Nonetheless, there is significant similarity outside of the AAA module between proteins with related functions [3]. It seems likely that the AAA module performs a function that is localized or adapted to different tasks by the non-AAA module regions of the molecules. The function of the AAA module is not known, but many AAA proteins are implicated in alteration of protein conformation, either reversible (chaperone-like activity, protein complex disassembly) or irreversible (unfolding before proteolysis) [1].

The 26S proteasome is a complex macromolecular assembly important for regulated protein degradation in eukaryotic organisms as well as archaebacteria and some eubacteria (for reviews, see [7,8]). Most substrates are targeted for degradation by components of the ubiquitin system through the covalent addition of a multiubiquitin

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chain [9]. The 26S proteasome is assembled from the 20S proteolytic and 19S regulatory sub-complexes [10]. The eukaryotic 20S proteasome contains 14 different subunits arranged into four stacked, seven-member rings that form a hollow cylinder [11]. The two inner rings are each composed of seven different β-type subunits, some of which have inward facing protease active sites, and two outer rings are each made up of seven different α-subunits [11]. The proteolytic active sites face the interior of the cylinder and are thus sequestered from the cellular environment, defining the proteasome as a 'self-compartmentalizing' protease [7,12].

In the 20S proteasome of *Thermoplasma* there is a 1.3 nM pore at each pole of the 20S cylinder [13]. This is sufficient to allow entry of peptides and unfolded proteins. In the *Saccharomyces cerevisiae* proteasome this pore is closed by overlapping loops of the α-type subunits [11]. Therefore, the pore must be opened before substrate can gain entry. Consistent with this, the *S. cerevisiae* 20S proteasome is usually purified in a 'latent' state that has very little peptidase activity [14,15]. The peptidase activity can be activated by treatments such as the addition of a low concentration of SDS, which might disorder the loops closing off the pore and thus allow access the interior of the cylinder [14,16]. While the SDS-activated complex is competent to degrade peptides, it can not degrade proteins. Recently, Groll and colleagues demonstrated that deletion of the N-terminal tails of the yeast 20S α-type subunits rendered the proteasomes fully active in vivo for both peptidase and protease activity [17].

Protein degradation by the proteasome requires assembly of the 20S with the 19S regulatory complex to form the 26S holocomplex [14]. The holocomplex is activated for peptidase activity and is capable of degrading Ub-conjugated proteins, a process that requires the hydrolysis of ATP [8]. The 19S regulatory complex of yeast is made up of at least 17 different subunits with a total molecular weight of approximately 700 kDa [14]. These subunits include six different ATPases of the AAA family. The genes for each of these ATPases are essential for the growth of yeast. The six ATPases are assumed to be responsible for the known ATP dependence of several proteasome functions. First, the association of the 19S and 20S complexes to form the 26S proteasome is ATP dependent [10]. Second, assembly of the 26S proteasome activates the peptidase activity of the 20S proteasome, possibly by opening the closed axial pore [18]. Rearrangement of the α-subunit loops to open the pore might be an ATP dependent action. Third, degradation of proteins by the proteasome requires ATP, presumably because substrates must be unfolded so that they can pass through the opened axial pore [19]. Fourth, ATP hydrolysis might be required for translocation of the unfolded proteins into the interior of the 20S and this activity might be linked to unfolding. There are no direct biophysical experiments proving a direct link between any of these activities and the ATPase activity of any specific AAA proteins. It is not clear if all of the ATPases are involved in each of these putative activities, or if there might be differentiation of functions between the ATPases.

To test the idea that different AAA ATPases in the 19S complex have different activities it is necessary to ablate the activity of just one of the ATPases and assay the functions of 26S complex lacking that activity. A simple approach would be to genetically delete the gene in question. However, all of the AAA proteins in the proteasome are essential for growth. A second strategy is to make mutations expected to inactivate the protein's function but not disrupt its overall structure. However, mutations in the proteasomal ATPases expected to dramatically reduce their ATPase activity proved lethal in four out of six cases [20]. In such cases, temperature sensitive (ts) mutations are often useful. However, these can be difficult to find, and it is often not clear if the shift in temperature results in a loss of activity, complete loss of structural integrity of the protein, or dissolution of the protein complex.

An ideal approach would be to find a drug that acts with high specificity on the active site of one of the ATPases. Since the AAA protein modules are very similar in sequence, especially in the nucleotide binding site [3], finding such a drug seems unlikely. An alternative approach is to make the protein of interest sensitive to a drug by a mutation that does not affect its activity in the absence of the drug. Toward this goal, we report a novel approach in which we are able to selectively sensitize individual ATPases to alkylation by NEM. We have used this approach to determine the contribution of individual ATPases to overall proteasomal function.

2. Results

Our approach to selective inactivation of individual proteasomal ATPases was suggested by a comparison between two AAA ATPases which are functional orthologs. NSF is a AAA protein involved in membrane fusion. As its name suggests, NSF (*N*-ethylmaleimide sensitive fusion protein) is exquisitely sensitive to the sulfhydryl modifying agent *N*-ethylmaleimide (NEM) and this property led to its identification and isolation [21]. The yeast homolog of NSF, Sec18, can substitute for NSF in a mammalian vesicle fusion assay [21]. However, Sec18 is much less sensitive to NEM. While NSF in cell extracts is inhibited by exposure to 1 mM NEM for 15 min on ice, at least 5 mM NEM is required to inhibit Sec18 under the same conditions [22,21]. Since NEM is a sulfhydryl modifying reagent [23], it has been suggested that a cysteine in the active site of NSF is responsible for its sensitivity to NEM [24]. The corresponding position in Sec18 is a threonine instead of a cysteine (Fig. 1) perhaps accounting for the difference in NEM sensitivity between these two proteins. Of 237 AAA

Protein	Walker Motif	Inhibitory [NEM]*
NSF	GPPGCGKT	1 mM
Sec18	T	5-8 mM
All Yeast 19S AAA Proteins	T	X
Sug1 T193C	C	>X ?
Sug2 T226C	C	>X ?

Fig. 1. Strategy for selective inactivation of individual yeast AAA proteins in the proteasome. The sequence of the Walker A box (nucleotide binding loop) is listed. NEM sensitivities for NSF and Sec18 are as reported by Wilson et al. [21]. All of the AAA proteins in the yeast 26S proteasome have a threonine at the position occupied by a cysteine in NSF and by a threonine in Sec18. We hypothesized that replacement of this threonine with a cysteine would increase the sensitivity of these proteins to alkylation by maleimides.

proteins having the consensus GPPGXGKT in the Walker A box, 170 proteins (72%) have X = T, and 43 proteins (18%) have X = C. Therefore, either T or C can be tolerated at this position in this group of highly homologous AAA modules. All of the yeast proteasomal AAA ATPases have a threonine at this position. Therefore, we reasoned that we could sensitize these proteins to NEM without crippling their ATPase activity by mutating the conserved threonine to a cysteine in individual ATPases (Fig. 1).

Modeling of the Sug1 ATP binding pocket suggests that this is a reasonable strategy. The ATP binding pocket of Sug1 was modeled based on the crystal structure of the D2 AAA module of NSF bound to the non-hydrolyzable ATP analog AMP–PNP [25]. The substitution of cysteine for threonine could be accommodated without any gross structural change to the active site (Fig. 2). Further, alkylation of the cysteine with NEM is predicted to interfere with ATP binding (Fig. 2). Specifically, the NEM moiety occupies much of the binding site for the β- and γ-phosphates of the ATP molecule. Therefore, we hypothesized that a comparison of proteasome activity between NEM-treated mutant and wild-type (WT) strains should allow us to assess the consequences of blocking the ATP binding and hydrolysis activities of individual ATPases. Interestingly, the second position in the DExx box of NSF is an aspartate which positions two water molecules that, in turn, interact with the γ-phosphate and the Mg^{2+}. In Sug1 this residue is a longer glutamate that can be modeled to interact with the Mg^{2+} ion and the γ-phosphate oxygen directly.

To test this hypothesis, we produced a set of congenic strains that differed only in the identity of a single amino acid in the Walker A box of a proteasomal ATPase. Threonine to cysteine mutations were produced in two proteasomal AAA proteins, Sug1/Rpt6 [26,27] and Sug2/Rpt4 [16]. The mutant alleles were placed into their normal genomic locations by two step gene replacement to

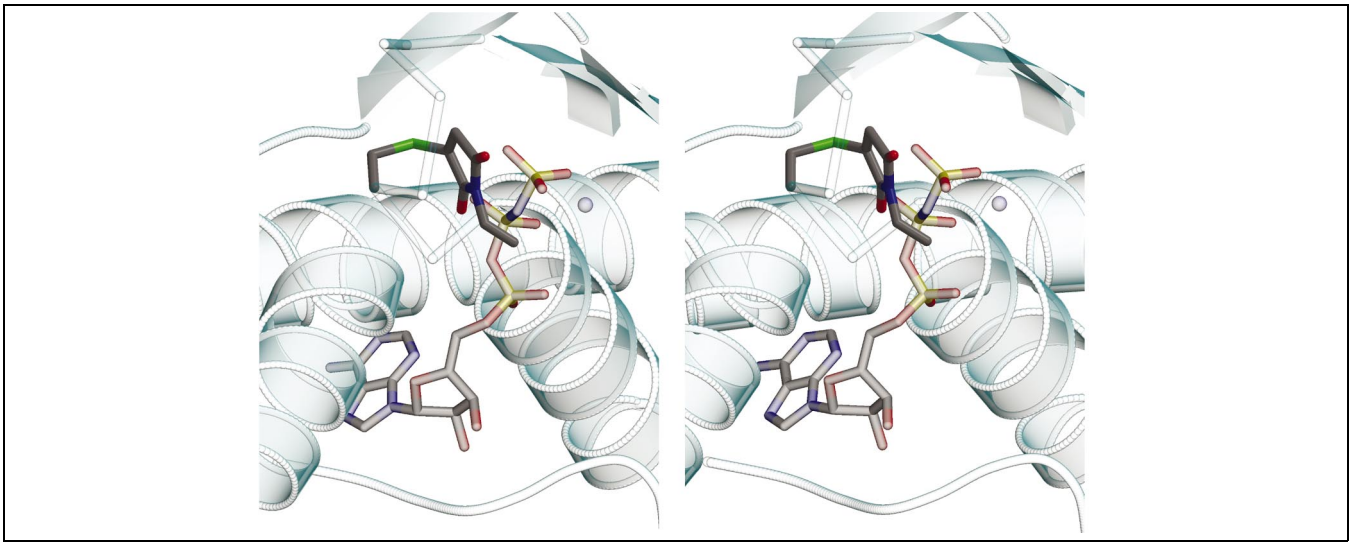

Fig. 2. Stereo view of the active site model for $Sug1^{T193C}$ with alkylation by NEM. The ATP binding sites of $Sug1^{T193C}$ was modeled based on the structure of the D2 AAA module of NSF [25] (see Section 2). The Sug1 Cα backbone trace is shown as a transparent ribbon diagram with the bound Mg^{2+} AMP–PNP (semi-transparent) and the cysteinyl-bound NEM (opaque) shown in a stick representation. Carbon atoms are shown in gray, oxygens in red, nitrogens in blue, phosphorus in yellow and sulfur is shown in green. This figure was prepared with Bobscript [37], Molscript [38] and Raster 3D [39,40].

Table 1
Peptidase activity of the 26S proteasome from *WT*, $SUG1^{T193C}$, $SUG2^{T226C}$, and $SUG1^{T193C}SUG2^{T226C}$ strains

Strain	Normalized peptidase activity	S.D. ($n=7$)
WT	1	
$SUG1^{T193C}$	1.08	0.04
$SUG2^{T226C}$	1.03	0.04
$SUG1^{T193C}SUG2^{T226C}$	1.09	0.05

Extracts of the indicated strains were assayed as described in Section 2. Results presented are the means of seven determinations.

produce haploid strains containing either the $SUG1^{T193C}$ or the $SUG2^{T226C}$ alleles which are otherwise WT. These strains grow as well as the congenic WT and exhibit no obvious phenotype (data not shown). These strains were mated and the resulting diploid was sporulated. Segregants from this cross were analyzed by PCR to identify WT, single mutant ($SUG1^{T193C}$ or the $SUG2^{T226C}$) and double mutant ($SUG1^{T193C}SUG2^{T226C}$) isolates so that a congenic set of strains was obtained. The Sug1 and Sug2 genes were amplified from genomic DNA isolated from these strains and sequenced to verify that only the desired mutations were present.

Many strains with defects in proteasomal proteolysis have obvious growth defects. Therefore, the growth characteristics of our panel of strains were analyzed. All four strains had the same doubling time at 30°C and 37°C in rich medium. They also had the same doubling time at 37°C in minimal medium (data not shown). Thus, these strains had no growth defect even under stressful growth conditions, suggesting that proteasome function is not grossly altered. To detect more subtle defects, the peptidase activity of the 26S proteasome from the mutant strains was tested. As shown in Table 1, there is no significant change in the peptidase activity of the 26S proteasome from either of the single mutant strains or the double mutant strain. In some experiments there was a slight increase in activity in the mutant strains, but this was not always observed.

To determine whether the substitution of cysteine for threonine in the nucleotide binding loop renders the mutant proteins more susceptible to alkylation with maleimides, we utilized 3-(*N*-maleimidopropionyl)-biocytin (biotin maleimide) [28]. This molecule is composed of maleimide attached to biotin through a long linker. We treated whole cell extracts with biotin maleimide, then quenched the reaction with excess DTT. Unreacted biotin maleimide was removed by gel filtration and the 26S proteasome complex was dissociated with guanidinium hydrochloride. Biotinylated proteins were then isolated by binding to streptavidin agarose. Bound proteins were separated by SDS–PAGE, and detected by Western blot. As can be seen in Fig. 3, Sug1 was preferentially alkylated by biotin maleimide in extract from the $SUG1^{T193C}$ strain as compared to extracts of the WT and the $SUG2^{T226C}$ strains. Conversely, Sug2 was preferentially alkylated in extracts from the $SUG2^{T226C}$ strain. WT Sug1 contains four cysteines and WT Sug2 contains two cysteines. The preferential labeling of Sug1 and Sug2 in extracts of the mutant strains is more dramatic than would be expected due to the addition of one additional cysteine if all cysteines are equally vulnerable to alkylation by biotin maleimide. Therefore, the cysteines introduced into the active site are uniquely accessible. This is not surprising, as the active site is by definition accessible to small molecules in solution. We cannot exclude that the mutations indirectly activate another residue as a nucleophile. However, this seems unlikely, and would not affect the usefulness of the method even if true. We conclude that Sug1 and Sug2 proteins carrying the threonine to cysteine mutations are preferentially targeted for alkylation by maleimide reagents.

To determine if alkylation of Sug1 and Sug2 has an effect on 26S proteasome function, we treated whole cell extracts from our panel of congenic strains with NEM. After excess NEM was quenched with DTT the peptidase activity of the 26S proteasome against a proteasome specific peptide substrate was assayed. Activity in this assay requires that the 26S proteasome remains intact. To assay the activity of the 20S proteasome alone, the 19S regulatory complex was dissociated from the 20S in the context of the whole cell extract. Activity was then assayed in the presence of 0.05% SDS which activates the latent 20S for peptidase activity.

The purified 26S proteasome has been demonstrated to be sensitive to NEM [29] and, as seen in Fig. 4A, NEM inhibits 26S peptidase activity in a dose dependent manner in all extracts. However, extracts of both single mutant

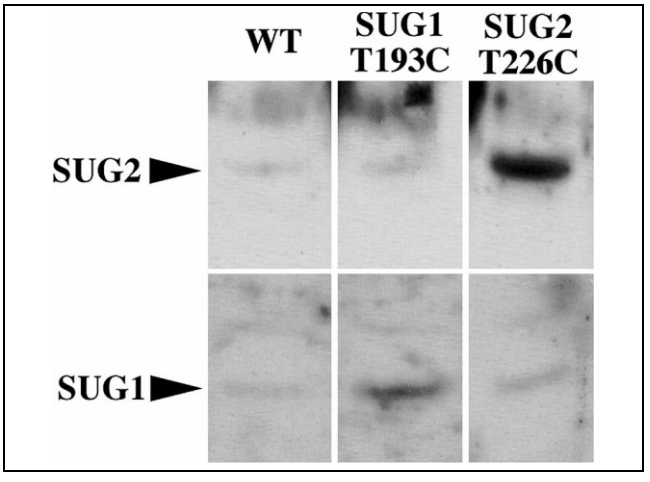

Fig. 3. Biotin maleimide differentially alkylates Sug proteins that have cysteines in their active sites. Extracts were treated with biotin maleimide and alkylated proteins were isolated under denaturing conditions using streptavidin agarose as described in Section 2. Western blots were probed with polyclonal antibodies specific for Sug1 or Sug2. Sug1 is preferentially alkylated by biotin maleimide only in the $SUG1^{T193C}$ strain and Sug2 is preferentially alkylated only in the $SUG2^{T226C}$ strain.

strains are more sensitive to NEM than WT, and extracts of the double mutant strain are more sensitive than the extracts of single mutant strains. To make the differential effect of NEM on extracts of the mutant strains more clear, the peptidase activity was normalized to the activity in the WT extract (Fig. 4B). As the dose of NEM increases from 0 to 2 mM, there is a progressively larger difference between activity of the WT and mutant extracts. However, after 2 mM there is no further increase in the differential effect, even though the absolute activity continues to decrease (compare Fig. 4A and B). We interpret this to mean that the mutant cysteines have been entirely alkylated by NEM after treatment with 2 mM NEM. Further alkylation by larger doses of NEM results in alkylation of residues which are common between the WT and mutant proteins. The sensitivity of the WT extract is not primarily due to NEM inhibition of the 20S proteasome. As can be seen in Fig. 4A, there is relatively little inhibition of SDS stimulated peptidase activity by NEM and there is no difference in inhibition between strains. Therefore, inhibition of 26S peptidase activity must be due to alkylation of cysteines normally present in proteins of the 19S complex besides Sug1 and Sug2. The critical point is that the sensitivity of the mutant strains to NEM is increased. Since the strains are otherwise identical, this increased sensitivity must be due to the presence of the variant cysteines, and thus likely due to the increased alkylation of Sug1 and Sug2.

Mutations in both Sug1 and Sug2 lead to a similar increase in NEM sensitivity of 25% compared to the WT extract. The double mutant has a sensitivity to NEM which is roughly the sum of the Sug1 and Sug2 effects, or approximately 50% inhibition of 26S peptidase activity compared to WT. Therefore, the functions of Sug1 and Sug2 are not redundant in activating the 20S proteasome for peptidase activity. An important point is that approximately 50% of 26S peptidase remains after both Sug1 and Sug2 are maximally alkylated. This seems reasonable, as only two of six ATPases have been differentially alkylated. However, this result, along with the additivity of the Sug1 and Sug2 effects, means that the activity of the AAA ATPases cannot be obligately coordinated. In other words, the other ATPases must be able to function to some degree even when Sug1 and Sug2 cannot.

At least two steps are necessary for the 19S to activate the 20S for peptide hydrolysis; binding to form the 26S complex and pore opening. Peptidase assays done in the absence of SDS measure the activity of intact 26S. If Sug1

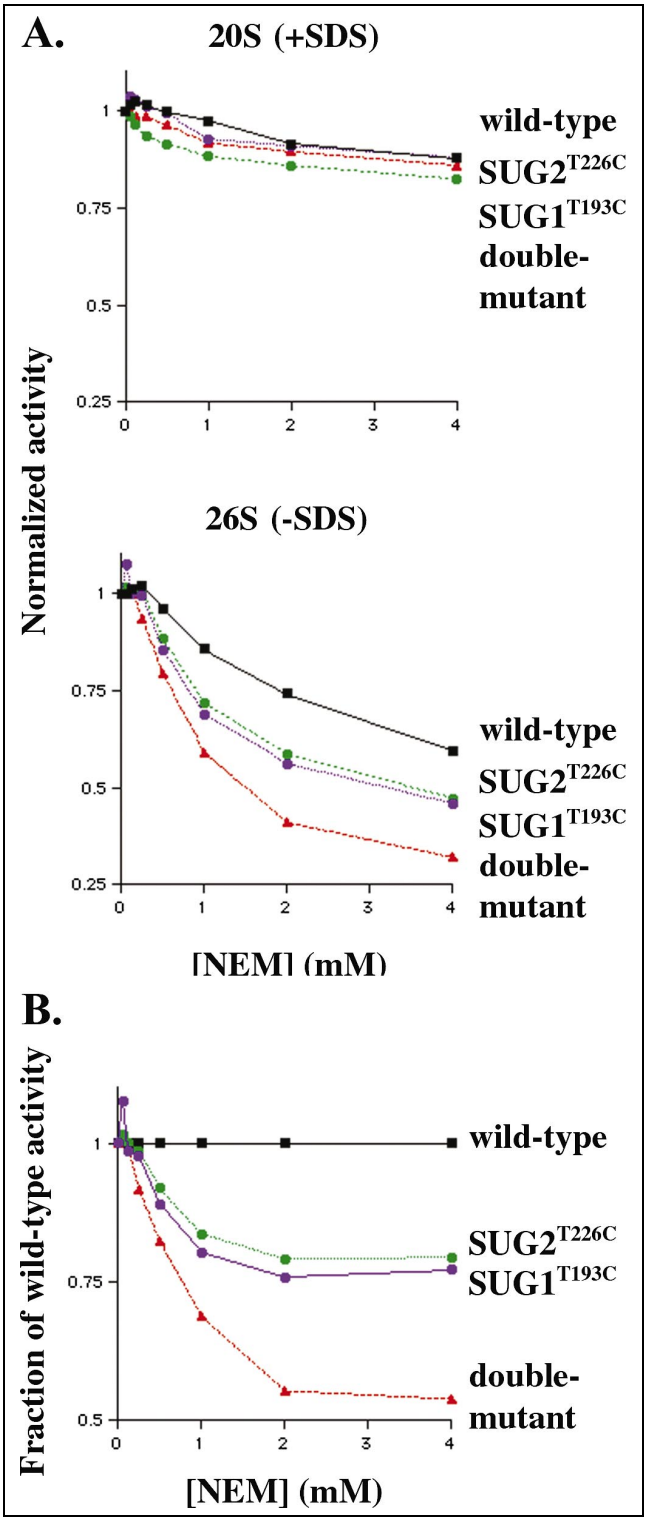

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Fig. 4. Sug1 and Sug2 have non-redundant functions in activation of the 20S proteasome peptidase activity. (A) Normalized peptidase activity of the 20S (+SDS) and 26S (−SDS) proteasomes. There is little effect of the concentrations of NEM used on the activity of the 20S proteasome activated by SDS. In contrast, non-SDS stimulated activity that requires activation by the 19S regulatory complex is inhibited in a dose dependent manner by NEM. Strains carrying threonine to cysteine mutations are more sensitive than WT strains. (B) Peptidase activity normalized to the activity of the WT strain. This is a different representation of the data in A. The $SUG1^{T193C}$ and $SUG2^{T226C}$ mutations have roughly equivalent effects and the effects of the two mutants seems to be additive in the $SUG1^{T193C}SUG2^{T226C}$ strain. The differential effect of NEM on the mutant strains is saturated at 2 mM NEM. At greater concentrations the inhibition of WT and mutant strains by NEM has the same dose dependence.

or Sug2 are important for the assembly and stability of the 26S complex, then NEM treatment of the mutant strains should result in destabilization of the complex compared to the similarly treated WT. In a kinetic assay we might expect the rate of peptide hydrolysis to decrease with time as the unstable 26S complex falls apart and thus loses peptidase activity. On the other hand, if Sug1 or Sug2 were involved in pore opening or maintaining the open position of the pore, then in a kinetic assay we would expect a change in the initial rate of hydrolysis because even stably assembled 26S proteasomes would have a decreased ability to admit substrate.

To determine if alkylation of Sug1 or Sug2 leads to different effects on 26S proteasome activity, we performed kinetic assays on extracts from the congenic panel of strains after treatment with NEM. The assays were performed as in Fig. 4 except that after the addition of treated extract to the substrate mix the concentration of product was measured every 30 s. Fig. 5A shows the progress of product accumulation for extracts from WT and mutant strains. Note that even in the WT extract that has not been treated with NEM the accumulation of substrate is not linear. Since substrate is not limiting even at the 30 min time point (data not shown), this implies that peptidase activity is lost during the course of the assay, possibly due to dissociation of the 26S proteasome into 19S and latent 20S complexes. This interpretation is supported by a graph of product accumulation by 20S proteasome activated by 0.05% SDS. The increase of product is linear and equivalent between strains in these experiments (data not shown).

In the absence of NEM treatment the mutant strains show a small increase in activity compared to the WT in this experiment. This was not always observed, and the differences were never large, consistent with the endpoint assay data shown in Fig. 4. After treatment with NEM the result is quite different (Fig. 5A,B). Even for the WT extract there is a more dramatic slowing of product accumulation at later time points than in the absence of NEM treatment. This suggests that alkylation of residues present in the WT proteins of the 19S proteasome destabilize the 26S complex. Consistent with the endpoint assay (Fig. 4) both single mutant extracts have similar decreases in the amount of product generated at the end of the reaction, and the double mutant demonstrates an additive effect. Interestingly, the Sug1 and Sug2 mutant extracts arrive at the same endpoint by different routes (Fig. 5A,B). The initial rate of product accumulation in the $SUG2^{T226C}$ extracts is the same as in the WT extract. However the rate of product accumulation decreases quickly so that by the end of the 30 min incubation the reaction rate is very slow. In contrast, the initial rate of product accumulation in the $SUG1^{T193C}$ extract is lower than that of the WT and $SUG2^{T226C}$ extracts. However, the decrease in rate of product accumulation during the incubation is not as dramatic as for the $SUG2^{T226C}$ ex-

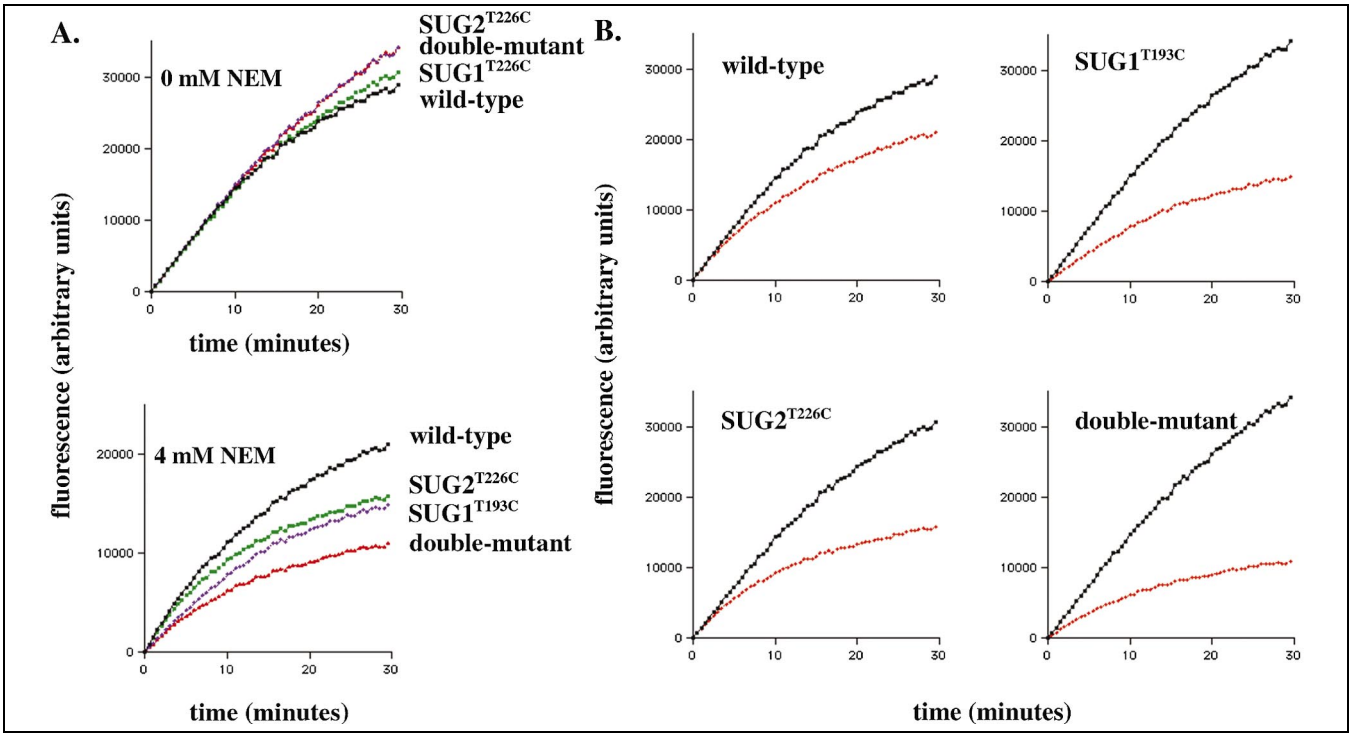

Fig. 5. Kinetic assays suggest different functions for Sug1 and Sug2 in 26S proteasome peptidase activity. (A) Accumulation of product over a 30 min time course in WT, single mutant, and double mutant strains either with or without NEM treatment. (B) Another representation of the data in A. In each panel the product accumulation for extract of a single strain is shown either with or without pretreatment with NEM. In each case the top trace is the untreated sample. See text for explanation.

tract, so that roughly the same amount of product is produced by the endpoint.

The kinetics of product accumulation in the $SUG1^{T193C}SUG2^{T226C}$ strain can be described as the sum of the two effects, with both a change in the initial rate as compared to the *WT*, and an accelerated decrease in rate of product accumulation during the course of the assay. Note that the relationship of the $SUG1^{T193C}SUG2^{T226C}$ trace to the $SUG1^{T193C}$ trace is similar to the relationship of the $SUG2^{T226C}$ trace to the *WT* trace. The initial rate of product accumulation is the same between the $SUG1^{T193C}SUG2^{T226}$ extract and the $SUG1^{T193C}$ extract, but then the two traces diverge due to an accelerated decrease in rate of product generation by the $SUG1^{T193C}SUG2^{T226}$ double mutant extract. Therefore, the presence of the $SUG2^{T226C}$ mutation leads to a decreased ability to maintain peptidase activity over the course of the reaction when present in either the *WT* or $SUG1^{T193C}$ backgrounds. These results suggest that alkylation of Sug1 or Sug2 leads to inhibition of 26S peptidase activity by different mechanisms.

3. Discussion

We have developed a strategy that allows individual ATPases to be inhibited within the intact 26S proteasome. Because the amino acid sequences of the proteasomal ATPases are so similar to each other, particularly within their ATP binding sites, our strategy does not rely on finding an inhibitor specific for each ATPase. Rather, we have sensitized individual ATPases to an inhibitor with limited specificity. Using this strategy, we have shown that Sug1 and Sug2 have non-redundant functions within the proteasome and can affect to proteasomal peptidase activity in different ways.

An analogy can be drawn between our chemical approach and the widely used genetic tact employing ts mutants. In both cases a change in the environment affects the activity of a mutant protein more than the WT. The ts strategy has been widely used, but it has several disadvantages. First, ts mutants can be difficult to find. It is not usually possible to predict which residues, when mutated, will make a protein sensitive to a change in temperature, so a genetic screen or selection must be designed. Second, it is often not clear what is happening to the mutant protein and any interacting partners upon temperature shift. In the best case, the mutant protein loses activity without being denatured or degraded. It is probably more often the case that the protein is reversibly or irreversibly denatured. In this case, interacting partners may also be lost to proteolysis, making it unclear whether the mutant protein is really responsible for the lost activity. In a subset of the ts strains, the already synthesized mutant protein may be unaffected by the temperature shift, but newly synthesized protein can not be correctly folded. In this case, the phenotype will take time to develop in vivo and the mutant cannot be used for in vitro studies. Finally, ts mutants often exhibit phenotypes even at the permissive temperature. Thus, the biological process under study does not behave normally even before the temperature shift, complicating the interpretation.

In contrast, our strategy of making 'chemical sensitive' mutants has several advantages. Based on evidence in the literature [21], we were able to predict an increase in sensitivity to maleimides for AAA proteins having a cysteine rather than a threonine at a highly conserved position within the nucleotide binding motif. Because the mutation was made in a region with a strongly predicted function (nucleotide binding), we could anticipate with reasonable confidence the consequences of alkylating the substituted cysteine residue. Because the cysteine was placed in the nucleotide binding pocket, it seemed likely that alkylation by NEM would prevent ATP binding but not destabilize the protein. Based on modeling of the $Sug1^{T193C}$ AAA domain and virtual alkylation of the cysteines by NEM (Fig. 2) it seems likely that the differences seen in NEM sensitivity between the WT and mutant extracts results from blocking ATP binding to Sug1 and/or Sug2, and not to a general disruption of the structure of the 19S regulatory complex. Finally, there is no detectable defect in proteasome function for any of the mutant strains in the absence of NEM. Thus, indirect effects due to defects in proteolysis during the growth of the mutant strains are unlikely. A disadvantage of this approach is that it is probably limited to proteins that, like the AAA proteins, have a residue that can be substituted with a cysteine in a critical site. Its use is also limited to use with extracts or purified proteins.

The use of NEM sensitizing mutations has allowed us to assess the contribution of individual proteins to the function of a very large protein complex. The 20S, which has a mass of approximately 700 kDa, can associate with either one or two 19S complexes [30,31,10]. The 26S proteasome contains at least 32 different polypeptides with a total mass of approximately 700 Da. The mass for the double capped complex is in excess of 2000 kDa [8]. In addition there are six different, yet highly similar, AAA ATPases which have nearly identical active sites ([14], and references therein). Therefore, determining the contribution of one ATPase to the function of the complex represented a significant challenge. It is becoming increasingly clear that many biological functions are performed by large protein machines [32]. A challenge for the future is to understand how each of their individual subunits contribute to the activity of the whole. Therefore, there will be a growing need for novel strategies like the one presented here.

The kinetic assays we report suggest that Sug1 and Sug2 have different roles in the function of the 26S proteasome. Sug2 may function in maintaining the interaction between the 19S and 20S proteasome. Because of the way the as-

says were performed, we cannot address whether Sug2 is also involved in the initial assembly of the 26S complex, but this seems likely. It was expected that proteasomal ATPases would be involved in assembly/stability of the 26S complex. The assembly process is known to be ATP dependent and the interaction is stabilized in the presence of ATP [14,10]. What is surprising is the apparently different degree of involvement of Sug1 and Sug2 in maintaining the 19S–20S interaction. The kinetic data suggest that the main effect of Sug1 alkylation is not to destabilize the 26S proteasome. A second requirement for ATPase activity may be opening of the axial pore. Immunoprecipitation experiments designed to investigate the relative amounts of 19S associated with 20S following NEM treatment have proved to be inconclusive, but we cannot rule out the possibility that the ATPase activity is involved in proteasomal assembly. It is also possible that allosteric activation of the protease active sites is required. The fact that treatments such as a low concentration of SDS can activate the 20S proteasome to the same degree as the 19S suggests that a conformational change in the 20S is sufficient to achieve activation. Therefore, Sug1 alkylation may either restrict access of substrate to the proteolytic active sites or result in failure to allosterically activate these sites. Consistent with our findings, Rubin et al. have shown that a variant of the AAA ATPase Rpt2 containing two mutations (rpt2RF) does not affect assembly of the 26S proteasome [20]. However, the assembled 26S proteasome containing rpt2RF has very little peptidase activity. This suggests that the activity of Rpt2 is not required for the ATP dependent assembly of the 26S proteasome, but has another role in activating the 20S for peptide hydrolysis. This mutant also points out an advantage of our approach over making mutants with effect on activity in the absence of any intervention. The rpt2RF allele was isolated after a conservative change in the Rpt2 Walker A box (K229R) proved lethal. An intragenic suppressor was then isolated (S241F), and it was the double mutant allele that was studied. Thus it is not clear what relationship the phenotype has to the ATPase activity of the mutant protein. ATPase activity may also be required for protein unfolding, which would allow for efficient processing through the axial pore, but the manner in which our assays were performed does not address that possibility.

The fact that our strategy works in the context of a crude extract suggests that it can be used for another type of experiment. While good inhibitors for the protease activity of the 26S proteasome exist (lactacystin, epoxomicin, peptide aldehydes), none are available to specifically inhibit the ATP dependent functions of the 19S regulatory complex. Therefore, it has been difficult to determine whether the 19S complex might have functions separate from assisting proteolysis by the 20S proteasome. Recently we used the approach described in this work to test the involvement of the proteasome in nucleotide excision repair (NER). A role for the proteasome in NER was suggested by the recently reported interaction with the NER protein Rad23 [33]. *WT* and $SUG1^{T193C}SUG2^{T226C}$ extracts competent for NER in vitro were treated in parallel with NEM. NER activity was more sensitive to NEM in extracts of the mutant than the WT strain. Surprisingly, NER in vitro is not sensitive to the 20S proteasome inhibitor lactacystin. Therefore, we were able to use the 'chemical sensitive' mutation strategy to show that the 19S regulatory complex functions independently of proteolysis in NER.

The inspiration for our strategy was the difference in NEM sensitivity between the human protein NSF and its yeast ortholog Sec18. Our findings demonstrate that the presence of a cysteine rather than a threonine at a conserved position within the active site of two AAA proteins can sensitize them to alkylation and inhibition by NEM. Therefore, it seems likely that this is the reason for observed differences in NEM sensitivity between NSF and Sec18. Although nearly all proteins are expected to be NEM sensitive at some level, certain AAA proteins are particularly sensitive. Our findings suggest a simple explanation for this phenomenon. Clearly, this strategy could be applied to the other four AAA proteins of the proteasome to define their roles in 26S function, but we propose that it may be more generally useful. Virtually any AAA protein could be studied. If the critical Walker A box position is occupied by a threonine, an NEM sensitive mutant can be created by substituting a cysteine. Alternatively, if this position is occupied by a cysteine a NEM resistant mutant can be created by substitution of a threonine. We hope that this approach will be useful for the study of the proteins containing the AAA domain, which have important roles in many cellular processes.

4. Significance

Functional studies regarding the properties of proteins is commonly performed through inhibition of known activities. Enzymatic inhibition has traditionally been achieved via the use of ts mutant proteins, or by the screening of chemical compounds for inhibitors to each protein of interest. However, the nature of the inactivation of ts mutant proteins is not well understood and these proteins are usually limited in availability. Therefore, we have developed a strategy for selectively inhibiting individual ATPases of the 19S regulatory particle of the proteasome. This strategy involves using site directed mutagenesis of the ATPase active site in order to sensitize the protein to the alkylating agent NEM. Using this technique, we find that the mutation of the protein does not affect the activity of the proteasome in the absence of NEM. In addition, our NEM-based strategy has allowed for the study of the contribution of individual subunits to the function of a large multiprotein complex. Kinetic assays performed suggest that the two proteins tested in this

study, Sug1 and Sug2, do not possess redundant functions in protein degradation. The AAA protein family consists of a large number of proteins that function in various processes within the cell. Considering the similarity in amino acid sequence of the proteins in the AAA family, this approach may be useful in the study of the involvement of the role of AAA proteins in cellular processes.

5. Materials and methods

5.1. Sequence comparisons and modeling

Sequences of AAA proteins for comparison were obtained from the AAA proteins database maintained by K.U. Froelich (available at http://yeamob.pci.chemie.uni-tuebingen.de/AAA). The closest AAA protein for which a crystal structure is available is of the D2 domain of NSF complexed with Mg^{2+}-AMPPNP [25,34]. The region around the ATP binding site is highly conserved and therefore could be used for modeling the Sug1 ATP binding site. Modeling was done by threading the Sug1 sequence with the T193C mutation into the NSF structure (PDB code: 1D2N). The most common rotamers were used initially. The model was examined and manually adjusted using O [35]. The only significant adjustment that was necessary was a rotation of lysine 195 for Sug1. In NSF, the amino group, Nζ of this lysine interacts with one of the γ-phosphate oxygens of ATP. This was rotated such that it interacts with a different oxygen on the same phosphate group. NEM was incorporated into the model by superimposing a cysteine-bound NEM derivative [*N*-2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC), from the crystal structure of phosphate binding protein (PBP) labeled with MDCC [36]. The coumaryl derivative bound to the nitrogen of maleimide was trimmed down to an ethyl group. Reaction of a cysteine with MDCC creates a new chiral center resulting in two diastereoisomers of the MDCC–PBP complex. Both are observed in the crystal structure. When superimposing the two cysteinyl-NEM moieties extracted from this, only one can be modeled into the ATP-binding region of Sug1 without clashing into the protein main chain.

5.2. Yeast strains

Site directed mutagenesis of Sug1 and Sug2 was performed with the Amersham Sculptor kit according to the manufacturer's instructions. The parental plasmids were pJS#159 and pSJR#87 which are pUC118 derivatives carrying the *SUG1* and *SUG2* genes respectively, including 5′ and 3′ untranslated regions. Oligo SJR#64 (5′-GGTTTTCCCGCAGCCAGGGGGG-3′) was used to make a mutation encoding T193C and insert a *Bgl*1 site. Oligo SJR#65 (5′-GTTTTACCACACCCGGGGGGACC-3′) was used to make a mutation encoding T226C and insert a *Sma*1 site. The plasmid produced was pSJR175 ($SUG1^{T193C}$). It was digested with *Eco*R1/*Kpn*1 to isolate an integration fragment. pSJR176 ($SUG2^{T226C}$) was digested with *Bst*E2/*Cla*1 for integration. Strains containing these mutant alleles were constructed using a two step gene replacement strategy. Strains Sc500 (*SUG1::URA3* pMTL-*SUG1*) and Sc530 (*SUG2::URA3* pMTL-*SUG2*) were transformed with the $SUG1^{T193C}$ and $SUG2^{T226C}$ fragments respectively. Strains Sc500 and Sc530 depend on expression of *SUG1* and *SUG2*, respectively, from the pMTL plasmids under control of the GAL promoter. Therefore, they survive only on galactose, which induces this promoter. After transformation, the strains were plated on glucose, which selects for transformants in which homologous integration occurs and SUG1 or SUG2 is now expressed from its own promoter. These strains were screened for correct integration by PCR and digestion of the amplification product at the restriction sites inserted for the purpose. The two single mutant strains produce were crossed and sporulated. The *SUG1* and *SUG2* genes were PCR amplified from individual spores. The PCR products were digested with *Bgl*1 (*SUG1*) and *Sma*1 (*SUG2*) to screen for single and double mutant segregants. The PCR products from candidate strains were sequenced completely to verify that only the desired mutations were present. In this way a set of four congenic strains were produced which had the $SUG1^{T193}$ allele and WT *SUG2*, the $SUG2^{T226}$ allele and WT *SUG1*, both mutant alleles, or two WT alleles.

5.3. Alkylation with biotin maleimide

Biotin maleimide (2-(*N*-maleimidopropionyl)-biocytin) was dissolved at a concentration of 10 mM in dimethylformamide (DMF). This solution was diluted with water to a final concentration of 5 mM, 50% DMF. Whole cell extracts in extract buffer (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 10% glycerol) were treated with a final concentration of 0.5 mM biotin maleimide (5% DMF) for 2 h before quenching the reaction with 5 mM DTT. Unreacted biotin maleimide was then removed by spun column gel filtration of the treated extracts through Centrisep columns (Princeton separations) equilibrated with extract buffer. The 26S proteasome complex was then dissociated by adding guanidinium hydrochloride to 8 M and incubating at 37°C for 10 min. The guanidinium chloride concentration was reduced to 4 M by the addition of an equal volume of extract buffer and biotinylated proteins were isolated by binding to streptavidin agarose (Boehringer Mannheim). The beads were extensively washed with 4 M guanidinium hydrochloride in extract buffer, then rinsed with 4 M urea. Bound proteins were released by autoclaving the beads in 2×SDS loading buffer, separated by SDS–PAGE, and detected by Western blot.

5.4. Measurements of proteasomal peptidase activity

NEM was dissolved at a concentration of 2 M in ethanol, then serially diluted in water to 40 mM NEM, 2% ethanol. Serial dilutions were then made in 2% ethanol. Extracts were treated with NEM for 15 min on ice before quenching unreacted NEM by addition of DTT to 5 mM. Peptidase activity assays were performed by diluting 10 μl of treated extract into 100 μl of Succ-LLVY-AMC substrate mix [16] Reactions were incubated at 30°C for 30 min. For assay of 20S activity, NaCl was added to 150 mM and the extracts were incubated overnight at 4°C to allow the 19S regulatory complex to dissociate from the 20S proteasome. Peptidase activity was then assayed as above except that the substrate mix contained 0.05% SDS. Kinetic peptidase assays were performed in a Tecan fluorescence plate reader. The reactions were started simultaneously by the addition of prewarmed substrate mix to wells containing treated extract with a multichannel pipetter. The plate was then incubated at 30°C with shaking between each measurement. The fluorescence in each well was measure every 30 s for 30 min.

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